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Award Number: DAMD17-00-1-0554

TITLE: The Functional Role(s) of Neurofibromin During Neural

Crest Cell Development

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REPORT DATE: August 2001

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command

Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;

Distribution Unlimited

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REPORT DOCUMENTATION PAGE

Form Approved OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

| 1. AGENCY USE ONLY (Leave blank) | 2. REPORT DATE | 3. REPORT TYPE AND | D DATES COVERED | | |
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The purpose of the studies to date have been to A)produce and maintain a colony of heterozygote neurofibromin (Nf+/-) in order to produce timed-pregnant neurofibromin knockout (Nf-/-) embryos. B) assess the ability of in vitro and in situ studies in order to quantitate the survival,proliferarion, migration and differentiation of neural crest cells derived from wild type (Nf+/+), Nf+/- and Nf-/- embryos. To achieve the aim in A, I have mated Nf+/- male mice with wild type mice to generate a colony of Nf+/- mice. This has proven more effective than directly mating male/female Nf+/- mice because Nf+/- have a greater tendency to cannibalize their litters versus wild-type females. With regards to B)I have firstly found it necessary to pair breeding mice during the day during a precise time window in order to accurately obtain embryonic day 9 (E9) time pregnant embryos. This is due to the narrow/transient time window of early NCC development in mice. Secondly, preliminary experiments strongly suggest that it is not feasible to the analysis neural crest development derived from individual embryos using a tissue culture paradigm but rather to use a in system whereby the NCC are analyzed directly in the embryo (in situ).

| 14. SUBJECT TERMS Neurofibromatosis, neurofibromin, neural | | | 15. NUMBER OF PAGES 7 |
|------------------------------------------------------------|------------------------------------------|-----------------------------------------|----------------------------|
| crest cells | | | 16. PRICE CODE |
| 17. SECURITY CLASSIFICATION OF REPORT | 18. SECURITY CLASSIFICATION OF THIS PAGE | 19. SECURITY CLASSIFICATION OF ABSTRACT | 20. LIMITATION OF ABSTRACT |
| Unclassified | Unclassified | Unclassified | Unlimited |

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Introduction

Neurofibromatosis type 1 (NF1) or von Recklinghausen's disease is a common autosomal dominant inherited disease involving aberrant growth of one or more neural crest cell (NCC)—derived cell types. The affected gene in NF1 encodes a potential tumor suppressor protein termed neurofibromin-1 (Nf-1, which is expressed by a number of cell-types including NCC. I hypothesize that in NF1, there is abnormal development of NCC thereby resulting in quantitative/qualitative perturbations in the generation of NCC-derived cell types. To date, there have been no systematic studies to determine whether or not aberrant development of NCC contributes to the formation of the above disease manifestations.

Brannan et al, have reported that autonomic ganglia are hyperplastic in early third trimester in NF1 knockout (NF1 ^{-/-}) mice. This finding indicates that PNS development is perturbed very early in these mice. It has been shown that PNS neurons (sympathetic neurons) derived from NF1 ^{-/-} mice exhibit neurotrophin-independent survival (Vogel et al., 1995). I have demonstrated that the survival/migration/proliferation and differentiation of rodent neural crest cells (which give rise to the glial and neuronal elements of PNS ganglia), are also modulated by trophic/growth factor support (Bannerman and Pleasure, 1993; Bannerman et al., 2000). I hypothesize that in NF1 ^{-/-} mice, growth factor dependency is also lost, resulting in increased clonal expansion/migration of NCC and neuronogenesis. To test this hypothesis, I will compare and contrast the survival, migration, proliferation and differentiation of NCC in normal, Nf-1^{+/-} heterozygote and Nf-1^{-/-} null mice. I also intended to use an in vitro assay system (however, see below) to pinpoint specific differences in growth factor independency between these NCC.

Body

The first priority of this study is to establish a healthy and viable colony of Nf+/-mice with which to generate Nf-/- embryos. For a number of reasons this has taken longer than anticipated. Firstly, I tried to generate Nf+/- mice quickly by mating Nf+/- males and females directly. However, I found that most of the Nf+/- females cannibalized their newborn offspring during the first day of birth. Subsequently, I have had more success in generating Nf+/- by mating Nf+/- males with wild-type females since the latter behave less 'quirky' and tend not to kill their progeny. Secondly, the breeding ability of the mice has been adversely affected by 1) an outbreak of mite infection in the breeding room and B) ongoing construction work here at Children's Hospital. The latter is causing major vibrations and perturbing the breeding habits of mice colonies in the animal facility,

including my own. The colony has also suffered attrition in Nf+/- females whereby females identified as pregnant by virtue of a vaginal plug, were sacrificed to provide E9 embryos and subsequently found not to be pregnant, or were not E9 embryos but younger or older than this date by half a day.

Early NCC development occurs during a narrow time frame and in the mouse this developmental window occurs at E9, where E8.5 and E9.5 are too early and too late respectively. This requires the investigator to know within several hours when the vaginal plug appears in the breeding female. Typically, mice are bred overnight and the assumption is made that copulation occurs at midnight. However, using this typical strategy, I have obtained few timed pregnant mice corresponding to precisely E9 but rather animals that are either E8.5 (bred soon after pairing) or E9.5 (bred a.m. the morning after pairing). To overcome this problem, I have paired animals at 10.00 a.m. and checked for plug formation at 1.00 pm and then at 4.00 pm. If no plug is found at these times, then the animals are separated and repaired the next day. This has successfully allowed me to obtained E9 embryos.

Despite the afore mentioned technical problems, I have produced sufficient Nf+/mice to undertake some preliminary studies relevant to the project. Given my considerable experience in studying NCC development in vitro, I therefore performed pilot experiments to study the proliferation rates of mouse NCC derived from wild-type, Nf+/-, and Nf-/- E9 embryos in culture. From these experiments, I was greatly concerned by the time involved in isolating, cleaning and harvesting of the initial starting tissue (spinal neuraxis). In a normal experiment to set numerous NCC cultures from a litter of embryos, the embryos are pooled and the dissection time for harvesting the embryonic tissue from the first to the last embryo of a litter is typically less than 20min. However, when dissecting and processing embryo tissues individually, and also obtaining tissue for genotyping (for the purpose of this study), the time between obtaining the first to the last embryo in the litter extended to approximately 120min. This variability in time interval most likely explains the large discrepancies in proliferation rate in NCC derived from embryos in same litter. For example, in one experiment the proliferation rate of NCC, as measured by the uptake of bromodeoxyuridine (BrdU), was found to be: 15.3, 24.7, 25.3, 31.9, 33.6, 41.7, 41.9, 46.9 in 8 of Nf+/- mice derived from the same litter. NCC derived from a single Nf-/- embryo in this litter had a BrDu uptake rate of 31.0. Compared to previous studies on NCC proliferation (Bannerman and Pleasure, 1993, Bannerman et al, 2000) the above wide variability in values is experimentally unacceptable. To circumvent this technical problem and moreover, analyze the development of NCC in the actual animal, I have developed a strategy to analyze NCC proliferation in situ. In this paradigm, the tissue from individual embryos is immediately fixed upon harvesting and therefore the integrity of the first embryo is preserved rather than being stored in tissue culture medium for up to 2 hours as part of the initial

dissection phase in the in vitro paradigm. The fixed embryos are then processed for cryostat sectioning. Individual embryos are serially sectioned onto a single slide and processed for immunohistochemistry using a mouse monoclonal antibody to AP2, which labels the nucleus of NCC, and rabbit polyclonal antibodies recognizing the phosphorylated form of histone 3 (a nuclear marker for cells undergoing mitosis). The use of the latter antibody to detect mitosis over the more traditional use of BrdU uptake, circumvents the need to perform a series of experiments to determine how long a pregnant mouse must be injected with concentrated BrdU in order to label dividing NCC in the embryos via placental transfer. This in situ analysis will be extended to identify NCC undergoing apoptosis in wild-type, Nf+/- and Nf-/-. These experiments will determine whether or not neurofibromin modulates NCC survival. In this analysis, the TUNEL procedure will be used in conjunction with AP2 immunohistochemistry to identify NCC undergoing apoptosis.

Key Research Accomplishments:

- 1) Established breeding colony to generate Nf-/- mice.
- 2) Set up daytime breeding protocol to accurately generate E9 embryos.
- 3) Tested viability of in vitro paradigm to analyze NCC development.
- 4) Developed in situ paradigm to replace inherently variable culture paradigm.

Reportable Outcomes:

The development of the in situ paradigm is novel and will be published in a manuscript along with data obtained from the above studies.

Conclusions:

The ability of an established tissue culture system to study whether Nf-1 modulates the proliferation of NCC has been evaluated and found to give erratically variable data. An alternative in situ paradigm has been developed and will be substituted for the tissue culture system, in the hope of providing more reproducible data to study the specific aim of this project. At the completion of this study it is hoped that I will have determined whether or not Nf-1 plays a biological role(s) in the development of NCC.

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Appendices: None